Stimulatory Effects of KPR-A148 on Osteoblast Differentiation and Bone Regeneration

Soomin Lim¹ · Ju Ang Kim¹ · Taeho Lee² · Doohyun Lee² · Sang-Hyeon Nam¹ · Jiwon Lim¹ · Eui Kyun Park¹

Received: 22 May 2019 / Revised: 11 June 2019 / Accepted: 12 June 2019 / Published online: 17 July 2019
© The Korean Tissue Engineering and Regenerative Medicine Society 2019

Abstract

BACKGROUND: Xanthine derivatives have been used to treat a variety of medical conditions including respiratory disease and neural degeneration. However, few studies have reported their effects on bone regeneration. Therefore, we investigated the effects of KPR-A148, a synthetic xanthine derivative on osteoblast differentiation *in vitro* and bone regeneration *in vivo*.

METHODS: The cytotoxicity of KPR-A148 was evaluated using MC3T3-E1 cells by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltertrazolium bromide assay. The effects of KPR-A148 on osteoblast differentiation were examined by alkaline phosphatase staining, Alizarin red S staining, and real-time PCR of osteoblast differentiation marker genes. To investigate the effects of KPR-A148 on *in vivo* bone regeneration, a KPR-A148-containing collagen sponge was implanted into a mouse calvarial defect and KPR-A148 was injected twice, weekly. Bone regeneration was evaluated quantitatively by micro-CT and qualitatively by hematoxylin and eosin, as well as Masson's Trichrome staining.

RESULTS: KPR-A148 did not show toxicity in the MC3T3-E1 cells and promoted osteoblast differentiation in a concentration-dependent manner. 10 μ M of KPR-A148 showed the most significant effect on alkaline phosphatase staining and matrix mineralization. KPR-A148 increased the expression of osteoblast marker genes in both the early and late stages of differentiation. In addition, KPR-A148 significantly induced new bone formation in the calvarial defect model.

CONCLUSION: These results demonstrate that KPR-A148 strongly induces osteoblast differentiation and new bone formation. Therefore, it could be used as a potential therapeutic agent for regenerating bone following its destruction by disease or trauma.

Keywords Osteoblast · Bone regeneration · Xanthine derivative · Collagen sponge

Soomin Lim, Ju Ang Kim and Taeho Lee have contributed equally to this work as first authors.

✉ Eui Kyun Park
epark@knu.ac.kr

¹ Department of Oral Pathology and Regenerative Medicine, School of Dentistry, Institute for Hard Tissue and Bio-tooth Regeneration (IHBR), Kyungpook National University, 2177 Dalgubeol-daero, Jung-gu, Daegu 41940, Republic of Korea

² College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 41566, Republic of Korea

1 Introduction

Bone defects arise from a variety of causes including infection, trauma, and tumors. Following appropriate treatment, spontaneous healing can occur in most bone defects under physiological conditions, however, large defects may not be completely healed due to their size and an unfavorable biomechanical environment. Hence, therapeutic strategies using bone graft substitutes are required for bone regeneration [1–3]. One strategy to reconstruct large bone defects is the implantation of scaffolds containing bioactive molecules such as growth factors,

peptides, and small molecules [4]. These biomolecules recruit osteoblasts and mesenchymal stem cells to the defect area and stimulate their differentiation into mature osteoblasts, which form a mineralized tissue in the defect area by incorporating hydroxyapatite into the newly formed organic matrix [5, 6]. Since growth factors and peptides have effective osteoinductive potential, they have been widely used for bone tissue engineering. However, they have a short half-life when administered *in vivo*, resulting in inefficient bone regeneration [7, 8]. For this reason, chemical compounds with low molecular weight and greater stability have been proposed as an alternative inducer for treating bone defects [9–11].

Xanthine derivatives are low-molecular-weight metabolites produced by the purine degradation pathway in animals and plants [12]. Naturally occurring xanthine derivatives such as caffeine, theophylline, and theobromine have been used as adenosine receptor antagonists [13] and bronchodilators [14]. Caffeine (1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione) has been used as a central nervous system stimulator [15] and for the treatment of apnea in premature infants [16]. Theophylline (dimethylxanthine-1,3-dimethyl-7H-purine-2,6-dione) is a secondary bronchodilator used for the treatment of asthma and chronic obstructive pulmonary disease [17]. Propentofylline (HWA285, 3-methyl-1-(5-oxohexyl)-7-propylpurine-2,6-dione), a synthetic xanthine derivative protects against nerve cell damage found in vascular dementia and Alzheimer's disease [18]. Recently, one of the natural xanthine derivatives, theobromine (3,7-dimethyl xanthine, 3,6 dimethyl purine-2,6-dione) was reported to have positive effects on osteoblast differentiation and skeletal development. Theobromine exposure on human mesenchymal stem cells promoted osteogenesis and the rate of mineralization. Also, oral administration of theobromine via dietary food from pregnancy until postnatal days accelerated the bone development in rats [19]. Pentoxifylline [3,7-dimethyl-1-(5-oxohexyl) purine-2,6-dione], a synthetic xanthine derivative, has also been reported to have a positive effect on bone homeostasis. Oral administration or subcutaneous injection of pentoxifylline for several weeks, increased the bone mass and bone strength under normal and pathological conditions [20–22]. These previous reports suggest that some of the synthetic xanthine derivatives have positive effects on bone regeneration. Therefore, we synthesized new xanthine derivatives and screened their ability to stimulate osteoblast differentiation. We found that one compound, KPR-A148 had a significant effect on osteoblast differentiation *in vitro* and bone regeneration *in vivo*.

2 Materials and methods

2.1 Cell culture and reagents

MC3T3-E1 cells, an osteoblast precursor cell line derived from the *Mus musculus* (mouse) calvaria [23], were purchased from ATCC (Manassas, VA, USA; CRL-2593), and used to analyze the expression of specific genes involved in osteoblast differentiation in response to KPR-A148. The cells were cultured in α -MEM (without ascorbic acid) containing 10% FBS, and osteoblast differentiation was induced using osteogenic media (OSM), containing ascorbic acid (50 μ g/ml), and β -glycerophosphate (10 mM). Ascorbic acid, β -glycerophosphate, Alizarin red S, and alkaline phosphatase (ALP) kit were purchased from Sigma-Aldrich (St Louis, MO, USA). KPR-A148 was synthesized as previously described [24], and the chemical structure of KPR-A148 [3-methyl-1,7-diphenyl-8-(propylthio)-1H-purine-2,6(3H,7H)-dione] has a phenyl group at N1 (nitrogen number 1) and N7, and a propylthio group at C8 (carbon number 8) in the xanthine structure (Fig. 1A). KPR-A148 was dissolved in dimethyl sulfoxide (DMSO; $\geq 99.7\%$, Sigma-Aldrich, St Louis, MO, USA).

Fig. 1 Chemical structure of KPR-A148 and MTT assay. **A** Chemical structure of KPR-A148. **B** KPR-A148 attenuated cell proliferation during osteoblast differentiation. MC3T3-E1 cells were cultured in OSM with or without 5, 10, 20, and 30 μ M of KPR-A148 for 4 days and MTT assay was performed

2.2 Cell viability assay

The viability of cells in response to KPR-A148 was measured using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St Louis, MO, USA). MC3T3-E1 cells were seeded into a 96-well plate at a density of 7×10^3 cells. The next day, media was changed to OSM with DMSO or KPR-A148. After 4 days, the supernatant was removed and 100 μ L of MTT solution (1 mg/mL) was added to each well. The plate was incubated at 37 °C for 1 h to allow the formation of formazan crystals. The purple formazan crystals were dissolved with DMSO, and then the optical density (OD) was measured at 570 nm with an ELISA reader (BioRad, Hercules, CA, USA).

2.3 Alkaline phosphatase staining

MC3T3-E1 cells were seeded in a 96-well plate at a density of 1.6×10^4 cells/well. The next day, the cells were treated with DMSO or KPR-A148 in the presence of OSM for 7 days. After the media was removed, the wells were washed twice with phosphate buffered saline (PBS), and then fixed at room temperature for 30 s. After washing with distilled water, diazonium solution (sodium nitrite: FRV-alkali line: distilled water: naphthol AS–BI alkali solution = 1:1:45:1) was treated at room temperature for 15 min. The plate was washed with distilled water and dried. ALP kit (Sigma-Aldrich, St Louis, MO, USA). was used for staining.

2.4 Alizarin red S staining

Mineralization was determined by Alizarin red S staining. MC3T3-E1 cells were seeded at a density of 1×10^5 cells per well in a 4-well plate. Next day, the culture medium was changed to OSM with DMSO or KPR-A148. After 4 weeks, Alizarin red S staining was performed. The MC3T3-E1 cells were washed with $1 \times$ PBS and fixed in 70% ethyl alcohol for 10 min at room temperature. After rinsing with distilled water, the cells were stained with Alizarin red S (40 mM, pH 4.2) for 10 min.

2.5 Quantitative real-time PCR

MC3T3-E1 cells were cultured in OSM, with DMSO or KPR-A148 for the indicated time. Total RNA was extracted using TRI-solution (Bioscience, Seoul, Korea) and then cDNA was synthesized from 1 μ g of RNA, using Superscript II (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed in a LightCycler 1.5 system (Roche Diagnostics, Rotkreuz, Switzerland), using SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan). The amplification conditions included an initial denaturation

step at 95 °C for 30 s; followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The mouse primers and conditions used for PCR are as follows: *Runx2*, 5'-GCA GTT CCC AAG CAT TTC AT-3' and 5'-CAC TCT GGC TTT GGG AAG AG-3'; *Alp*, 5'-GCT GAT CAT TCC CAC GTT TT-3' and 5'-CTG GGC CTG GTA GTT GTT GT-3'; *Colla1*, 5'-GAG CGG AGA GTA CTG GAT CG-3' and 5'-TAC TCG AAC GGG AAT CCA TC-3'; *Oc*, 5'-ATG AGG ACC ATC TTT CTG CTC-3' and 5'-GTA GCG CCG GAG TCT GTT C-3'; *Bsp*, 5'-AAA GTG AAG GAA AGC GAC GA-3' and 5'-GTT CCT TCT GCA CCT GCT TC-3'; *Gapdh*, 5'-ATG ACA TCA AGA AGG TGG TG-3' and 5'-CAT ACC AGG AAA TGA GCT TG-3'; *Osterix (Osx)*, 5'-ACC AGG TCC AGG CAA-3' and 5'-GCA GTC GCA GGT AGA-3'.

2.6 Animal model

Animal experiments were performed in accordance with the guidelines approved by Kyungpook National University (No. 2017-0074). The bone regeneration by KPR-A148 was evaluated in 6-week-old male mice using a calvarial defect model. The mice were anesthetized with an intraperitoneal injection of a mixture of tribromoethanol (Avertin) at a dose of 22 μ L/g body weight. The head was shaved and cleaned with PBS and a povidone-iodine solution. The calvarial bone was exposed by a midline incision in the parietal region, and the overlying periosteum was elevated to expose the bony area. Calvarial defects were made using a trephine (1.8 mm diameter) bur (MCTBIO, Yongin, Korea) in a slow dental handpiece (Surgic XT, Nakanishi, Japan). Sterile PBS was used for irrigation during drilling. Collagen sponge (4 mm diameter; SpongeCol®, Advanced BioMatrix, San Diego, USA) soaked with DMSO or KPR-A148 was implanted into the defect site. Finally, the defects were covered with periosteum and the skin was sutured with 5–0 vicryl (AILEE Co., Busan, Korea). We injected 5 μ L with the indicated concentration of 1% DMSO or 20 and 100 μ M KPR-A148, twice a week, directly into the defect area using an insulin syringe. The mice were divided into three groups: collagen sponge with DMSO (n = 4), collagen sponge with 20 μ M of KPR-A148 (n = 5), and collagen sponge with 100 μ M of KPR-A148 (n = 4).

2.7 Micro-computed tomography (CT) and histomorphometric analysis

The mice were sacrificed 4 weeks after surgery by cervical dislocation. The calvaria was removed and fixed in 10% neutral buffered formalin solution for 24 h. Micro-CT scan of the mouse calvaria was performed using a Skyscan 1272

scanner (Bruker-microCT, Konich, Belgium). Scanning was carried out at 70 kV, 142 uA, with an Al 0.5 mm filter, and the exposure was set to 537 ms. The scanning resolution was 10 μm per voxel with a 1632×1092 pixel matrix. The CTvol software (Bruker-microCT, Konich, Belgium) was used for 3D visualization of the reconstructed section. The sagittal suture area was measured on digitally recorded projections of micro-CT 3D images using CTAn image analysis software (Bruker-microCT, Konich, Belgium). For histological analysis, the samples were decalcified in 0.5 M EDTA for 2 weeks and then embedded in paraffin. Histological sections of 5 μm thickness were processed for Hematoxylin/eosin (H&E) and Masson's Trichrome staining.

2.8 Statistical analysis

The quantitative data are presented as mean $\pm$ standard deviation (SD). The statistical analysis was performed by the two-tailed Student's *t* test. A *p* value of less than 0.05 was considered as statistically significant.

3 Results

3.1 Cytotoxicity of KPR-A148 on MC3T3-E1 cells

To assess the cytotoxic effects of KPR-A148 during osteoblast differentiation, we cultured MC3T3-E1 cells in OSM with 0, 5, 10, 20, and 30 μM of KPR-A148. Four days after treatment, MTT assay was performed to evaluate the cytotoxicity. KPR-A148 attenuated the growth of MC3T3-E1 cells slightly at high concentrations (20 and 30 μM), however, there was no statistical significance between all the groups (Fig. 1B). This result indicated that KPR-A148 may not induce toxic effects during osteoblast differentiation of MC3T3-E1 cells even at high concentrations.

3.2 Effect of KPR-A148 on osteoblast differentiation

To determine the effects of KPR-A148 on osteoblast differentiation, MC3T3-E1 cells were cultured in OSM with or without various concentrations of KPR-A148. First, we performed staining for ALP, a very early marker of osteoblast differentiation. As shown in Fig. 2A, KPR-A148 stimulated the ALP activity in a dose-dependent manner and showed the highest effects at a concentration of 10 μM . At higher concentration (20 μM), the ALP activity was slightly decreased. These findings indicate that treatment with 10 μM of KPR-A148 may be the optimal condition for osteoblast differentiation.

Next, to investigate the effects of KPR-A148 on mature osteoblast differentiation, we examined the mineralization

of MC3T3-E1 cells. Cells were treated with KPR-A148 at 10 and 20 μM during osteoblast differentiation. After 4 weeks, Alizarin red S staining was performed to detect calcium deposition in the matrix. As shown in Fig. 2B, KPR-A148 significantly promoted the matrix mineralization of MC3T3-E1 cells at 10 and 20 μM concentrations, with 10 μM showing the most effect on mineralization. These results suggest that KPR-A148 has a strong stimulatory effect on osteoblast differentiation.

3.3 Effect of KPR-A148 on osteoblast marker gene expression

To confirm the stimulatory effects of KPR-A148 on osteoblast differentiation, we analyzed the expression of osteoblast marker genes using real-time PCR (Fig. 2C). First, we analyzed the osteoblast-specific transcription factors, *Runx2* and *Osx*. *Runx2* expression in KPR-A148-treated cells was 1.5 times higher than in control cells, and *Osx* gene was significantly induced almost two times by KPR-A148 at 4 and 8 days. However, the increase in *Runx2* expression in the KPR-A148-treated cells was not found to be significant. Next, we analyzed the expression of early osteoblast markers, *Alp* and type I collagen (*Colla1*). *Alp* expression generally reached the highest level at the end of proliferation period then decrease [25]. KPR-A148 significantly induced *Alp* expression, over 1.5 times higher than the control group at all the indicated time points, this indicates KPR-A148 effectively promote inorganic phosphate generation for mineralization at late stage of differentiation. *Colla1* expression was also increased in the KPR-A148 group during osteoblast differentiation. Lastly, we analyzed the late markers of osteoblast differentiation, including bone sialoprotein (*Bsp*) and osteocalcin (*Oc*), both of which were markedly elevated in KPR-A148-treated cells during differentiation. *Bsp* expression in the KPR-A148 group was increased 1.8 and 2 times at 4 and 8 days, respectively. *Oc* expression in the KPR-A148 group was increased over fivefold at 4 and 8 days. These results suggest that KPR-A148 strongly promotes the expression of osteoblast marker genes during differentiation.

3.4 Micro-CT analysis of calvarial bone regeneration by KPR-A148

To investigate the effects of KPR-A148 on bone regeneration *in vivo*, the collagen sponges soaked with DMSO, 20 μM , or 100 μM of KPR-A148 were implanted in the mouse calvarial defect, and KPR-A148 was directly injected into the implanted region, twice a week. After 4 weeks, the calvarial bone regeneration was evaluated by micro-CT. As shown in Fig. 3A, the control group showed only a little bone growth from the marginal area of the

Fig. 2 KPR-A148 stimulates osteoblast differentiation of MC3T3-E1 cells. MC3T3-E1 cells were cultured in OSM in the presence of various concentrations of KPR-A148. **A** Alkaline phosphatase activity was evaluated by ALP staining at 7 days of culture. **B** Matrix mineralization was assessed by Alizarin Red S staining after 4 weeks of differentiation. **C** MC3T3-E1 cells were cultured in OSM with or without 10 μ M of KPR-A148 for 1, 4, and 8 days. The mRNA expression of *Runx2*, *Osx*, *Alp*, *Col1a1*, *Bsp*, and *Oc* was evaluated using real-time quantitative PCR. GAPDH was used as an internal control. * $p < 0.05$; ** $p < 0.01$ versus DMSO control

Fig. 3 Micro-CT and bone parameter analysis revealed increased bone regeneration in the calvarial defect mouse model following treatment with collagen sponge containing KPR-A148. Collagen sponge soaked with DMSO, 20 μ M, and 100 μ M of KPR-A148 was implanted in mouse calvarial defects. KPR-A148 was then directly injected into the defect area, twice a week. Four weeks after implantation, the calvaria was scanned using a micro-CT. **A** Representative images showing coronal and transaxial images of the calvarial defect.

B Quantitative analysis of bone parameters in KPR-A148-treated or non-treated calvaria. Bone volume (BV), bone volume per tissue volume (BV/TV), trabecular number Tb.N(1/mm), and trabecular thickness (Tb.Th) were analyzed. Collagen sponge with DMSO (n = 4), collagen sponge with 20 μ M of KPR-A148 (n = 5), and collagen sponge with 100 μ M of KPR-A148 (n = 4). * p < 0.05; *** p < 0.001 versus indicated group

defect. However, collagen sponge soaked with 20 μ M of KPR-A148 induced new bone formation not only at the defect edge but also in the central region of the defect. Collagen sponge soaked with a higher concentration of KPR-A148 (100 μ M) induced significantly higher bone formation in both the marginal and central area. Quantitative analysis of bone parameters also revealed gradually increased bone healing in the defect area by KPR-A148 in a dose-dependent manner (Fig. 3B). The percentage increase in bone volume (BV) was 255%, bone volume fraction (BV/TV) was 328%, trabecular number (Tb.N) was 144%, and trabecular thickness (Tb.Th) was 200% in the 20 μ M KPR-A148 group. At a higher concentration of KPR-A148 (100 μ M), all parameters including BV, BV/TV, Tb.N, and Tb.Th were increased by 3757%, 3894%, 1267%, and 400%, respectively.

3.5 Histological analysis of calvarial bone regeneration by KPR-A148

To confirm new bone formation, we performed a histological analysis of the KPR-A148-treated or non-treated calvarial defects. H&E staining showed that the newly formed osteoid tissue was present at the edge of the defect in all groups. While the control group showed poor osteoid formation in the central region, both 20 μ M and 100 μ M concentrations of KPR-A148 highly promoted new osteoid tissue formation at the center of the defect (Fig. 4A boxes). Moreover, the high concentration of KPR-A148 (100 μ M) showed higher mineralization in the newly formed bone compared to the other two groups (Fig. 4A). Masson's Trichrome staining also indicated that all three experimental groups have collagen connective tissues (blue),

Fig. 4 Histological analysis showed increased mature bone formation in the calvarial defect mouse model treated with collagen sponges containing KPR-A148. **A** Low magnification images (50 $\times$, upper panels) and high magnification images of the boxed region (200 $\times$, black, lower panels) of the hematoxylin and eosin (H&E)-stained defect area. **B** Low magnification images (50 $\times$, upper panels) and

high magnification images of the boxed region (200 $\times$, yellow, lower panels) of the Masson's Trichrome-stained defect area. (NB: new bone) The collagen sponge soaked with DMSO (control, $n = 4$), collagen sponge soaked with 20 μM of KPR-A148 (20 μM , $n = 5$) and collagen sponge soaked with 100 μM of KPR-A148 (100 μM , $n = 4$)

suggesting that the defect area undergoing new bone formation is not fully mature. However, the new bone areas showed more red stain in the high concentration of KPR-A148 (100 μM) group, indicating that the new bone is undergoing active mineralization (Fig. 4B). These data suggest that KPR-A148 treatment is able to induce effective bone regeneration *in vivo*.

4 Discussion

Various forms of natural xanthine derivatives from tea, coffee, cocoa, and chocolate, as well as synthetic forms, are used in pharmaceutical agents, such as cyclic nucleotide phosphodiesterase inhibitors and antioxidants [26, 27].

Despite the therapeutic potential of xanthine derivatives in various diseases, few studies have reported their effects on osteoblast differentiation or bone regeneration.

We have demonstrated that synthetic KPR-A148 induced osteoblast differentiation even at low concentrations of 10 μM or less (Fig. 2). A previous report showed that theobromine, a natural xanthine derivative, stimulated bone formation at a high concentration [19]. Although both xanthine derivatives have similar effects on osteoblasts, KPR-A148 is structurally distinct from theobromine, indicating that KPR-A148 is a new xanthine derivative capable of inducing osteoblast differentiation.

KPR-A148 significantly increased the gene expression of osteoblast markers, however, the expression of *Runx2* was not statistically significant (Fig. 2C). These results

suggest that *Runx2* may not play a critical role in the enhanced osteoblast differentiation by KPR-A148. *Runx2* is accepted as the most important master gene involved in osteoblast differentiation, however, some previous studies have reported *Runx2*-independent osteoblast differentiation [28, 29]. In particular, the increase in *Osx* and *Oc* expression by *Bmp2* in the *Runx2*-deficient cell line showed a very similar expression pattern as observed in this study. These results suggest that KPR-A148 may induce osteoblast differentiation by increasing the expression of *Osx* or *Oc* rather than the *Runx2* pathway.

Having established that KPR-A148 substantially stimulates osteoblast differentiation *in vitro*, its effects on bone regeneration *in vivo* was examined in a mouse calvarial defect model. We used collagen sponge as a carrier for KPR-A148 in the bone regeneration system *in vivo*. Generally, the administration dosage is much higher than that of *in vitro* experiment because chemicals are metabolized in the body. However, we tried to use as low amount of KPR-A148 as possible, and selected two concentrations (20 μ M and 100 μ M). In the first pilot experiment, KPR-A148 were soaked in the collagen sponge and implanted in the calvarial defects. According to our findings, bone regeneration was not clearly observed at 4 weeks after surgery (data not shown). As shown in Fig. 2, KPR-A148 affects not only the early stage but also the late stage of osteoblast differentiation. We supposed that the lack of clear bone regeneration *in vivo* may be because the simple soaking procedure probably allowed the KPR-A148 to dissolve out of the collagen sponge before giving rise to its effects. Therefore, we used collagen sponges soaked with KPR-A148 before implantation followed by KPR-A148 injection into the defect area, twice a week to minimize the disturbance of animal for 4 weeks and this procedure produced significant bone regeneration as shown in Figs. 3 and 4.

The evaluation of bone regeneration is a key step in assessing the osteoconductive and osteoinductive abilities of bone-stimulating biomaterials or biomolecules during bone regeneration. As shown in the micro CT data (Fig. 3), there was significant osteoinduction in the central region of the defect area where we directly injected KPR-A148 after surgery. Generally, calvarial defect healing occurs by bone growth from the defect edge (osteoconduction); however, the group receiving KPR-A148 injections exhibited osteoinduction in the central region of the defect area, indicating that prolonged administration of KPR-A148 is important for *in vivo* bone regeneration. The histological data also demonstrated a dose-dependent increase in newly formed bone in the KPR-A148-treated group (Fig. 4). Interestingly, we found that the newly mineralized bone had a few lacunae but no lamina, suggesting that the new bone is still undergoing maturation (Fig. 4). Our data

suggest that considering the duration of KPR-A148 activity on osteoblast differentiation and maturation, it would be more efficient to apply KPR-A148 to the bone substitute by incorporation of covalent bonds rather than surface soaking or injection for long term stimulation [30].

In summary, the synthetic xanthine derivative, KPR-A148, significantly stimulates osteoblast differentiation *in vitro* and increases bone regeneration in the bone defect model. These results suggest that the newly discovered synthetic molecule KPR-A148 may be used as a potential bone regeneration factor to repair the bone defects caused by trauma, tumors, surgery, or infections.

Acknowledgements This research was supported by the Bio and Medical Technology Development Program of the National Research Foundation (NRF) and funded by the Korean government (MSIT) (NRF-2017M3A9E4047244).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All animal experiments were approved by the committee on the care and use of animals in research at Kyungpook National University, South Korea, and conducted in accordance with the guidelines for the Care and Use of Laboratory Animals (2017-0074).

References

- Shivalkar S, Singh S. Solid freeform techniques application in bone tissue engineering for scaffold fabrication. *Tissue Eng Regen Med*. 2017;14:187–200.
- Kashte S, Jaiswal AK, Kadam S. Artificial bone via bone tissue engineering: current scenario and challenges. *Tissue Eng Regen Med*. 2017;14:1–14.
- Li Y, Chen SK, Li L, Qin L, Wang XL, Lai YX. Bone defect animal models for testing efficacy of bone substitute biomaterials. *J Orthop Translat*. 2015;3:95–104.
- Ho-Shui-Ling A, Bolander J, Rustom LE, Johnson AW, Luyten FP, Picart C. Bone regeneration strategies: engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives. *Biomaterials*. 2018;180:143–62.
- Chamieh F, Collignon AM, Coyac BR, Lesieur J, Ribes S, Sadoine J, et al. Accelerated craniofacial bone regeneration through dense collagen gel scaffolds seeded with dental pulp stem cells. *Sci Rep*. 2016;6:38814.
- Tollemar V, Collier ZJ, Mohammed MK, Lee MJ, Ameer GA, Reid RR. Stem cells, growth factors and scaffolds in craniofacial regenerative medicine. *Genes Dis*. 2016;3:56–71.
- Devescovi V, Leonardi E, Ciapetti G, Cenni E. Growth factors in bone repair. *Chir Organi Mov*. 2008;92:161–8.
- Pountos I, Panteli M, Lampropoulos A, Jones E, Calori GM, Giannoudis PV. The role of peptides in bone healing and regeneration: a systematic review. *BMC Med*. 2016;14:103.
- Aravamudhan A, Ramos DM, Nip J, Subramanian A, James R, Harmon MD, et al. Osteoinductive small molecules: growth factor alternatives for bone tissue engineering. *Curr Pharm Des*. 2013;19:3420–8.

10. Balmayor ER. Targeted delivery as key for the success of small osteoinductive molecules. *Adv Drug Deliv Rev.* 2015;94:13–27.
11. Kim JA, Yun HS, Choi YA, Kim JE, Choi SY, Kwon TG, et al. Magnesium phosphate ceramics incorporating a novel indene compound promote osteoblast differentiation in vitro and bone regeneration in vivo. *Biomaterials.* 2018;157:51–61.
12. Rosemeyer H. The chemodiversity of purine as a constituent of natural products. *Chem Biodivers.* 2004;1:361–401.
13. Müller CE, Jacobson KA. Xanthines as Adenosine Receptor Antagonists. In: Fredholm BB, editor. *Methylxanthines. Handbook of Experimental Pharmacology*, vol 200. Berlin: Springer; 2018. p. 151–99.
14. Cushley MJ, Holgate ST. Bronchodilator actions of xanthine derivatives administered by inhalation in asthma. *Thorax.* 1985;40:176–9.
15. Nehlig A, Daval JL, Debry G. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Res Brain Res Rev.* 1992;17:139–70.
16. Abdel-Hady H, Nasef N, Shabaan AE, Nour I. Caffeine therapy in preterm infants. *World J Clin Pediatr.* 2015;4:81–93.
17. Barnes PJ. Theophylline. *Am J Respir Crit Care Med.* 2013;188:901–6.
18. Rother M, Erkinjuntti T, Roessner M, Kittner B, Marcusson J, Karlsson I. Propentofylline in the treatment of Alzheimer's disease and vascular dementia: a review of phase III trials. *Dement Geriatr Cogn Disord.* 1998;9 Suppl 1:36–43.
19. Clough BH, Ylostalo J, Browder E, McNeill EP, Bartosh TJ, Rawls HR, et al. Theobromine upregulates osteogenesis by human mesenchymal stem cells in vitro and accelerates bone development in rats. *Calcif Tissue Int.* 2017;100:298–310.
20. Pal S, Porwal K, Khanna K, Gautam MK, Malik MY, Rashid M, et al. Oral dosing of pentoxifylline, a pan-phosphodiesterase inhibitor restores bone mass and quality in osteopenic rabbits by an osteogenic mechanism: a comparative study with human parathyroid hormone. *Bone.* 2019;123:28–38.
21. Vashghani Farahani MM, Ahadi R, Abdollahifar M, Bayat M. The effects of pentoxifylline administration on fracture healing in a postmenopausal osteoporotic rat model. *Lab Anim Res.* 2017;33:15–23.
22. Kinoshita T, Kobayashi S, Ebara S, Yoshimura Y, Horiuchi H, Tsutsumimoto T, et al. Phosphodiesterase inhibitors, pentoxifylline and rolipram, increase bone mass mainly by promoting bone formation in normal mice. *Bone.* 2000;27:811–7.
23. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol.* 1983;96:191–8.
24. Lee D, Lee S, Liu KH, Bae JS, Baek DJ, Lee T. Solid-phase synthesis of 1,3,7,8-tetrasubstituted xanthine derivatives on traceless solid support. *ACS Comb Sci.* 2016;18:70–4.
25. Beck GR Jr, Zerler B, Moran E. Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci U S A.* 2000;97:8352–7.
26. Boswell-Smith V, Cazzola M, Page CP. Are phosphodiesterase 4 inhibitors just more theophylline? *J Allergy Clin Immunol.* 2006;117:1237–43.
27. Vignoli JA, Bassoli DG, Benassi MT. Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: the influence of processing conditions and raw material. *Food Chem.* 2011;124:863–8.
28. Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem Biophys Res Commun.* 2003;309:689–94.
29. Liu T, Gao Y, Sakamoto K, Minamizato T, Furukawa K, Tsukazaki T, et al. BMP-2 promotes differentiation of osteoblasts and chondroblasts in Runx2-deficient cell lines. *J Cell Physiol.* 2007;211:728–35.
30. Kim JA, Choi YA, Yun HS, Bae YC, Shin HI, Park EK. Extracellular calcium-binding peptide-modified ceramics stimulate regeneration of calvarial bone defects. *Tissue Eng Regen Med.* 2016;13:57–65.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.